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Supplemental information

WNK1 is an assembly factor for the human ER membrane protein complex

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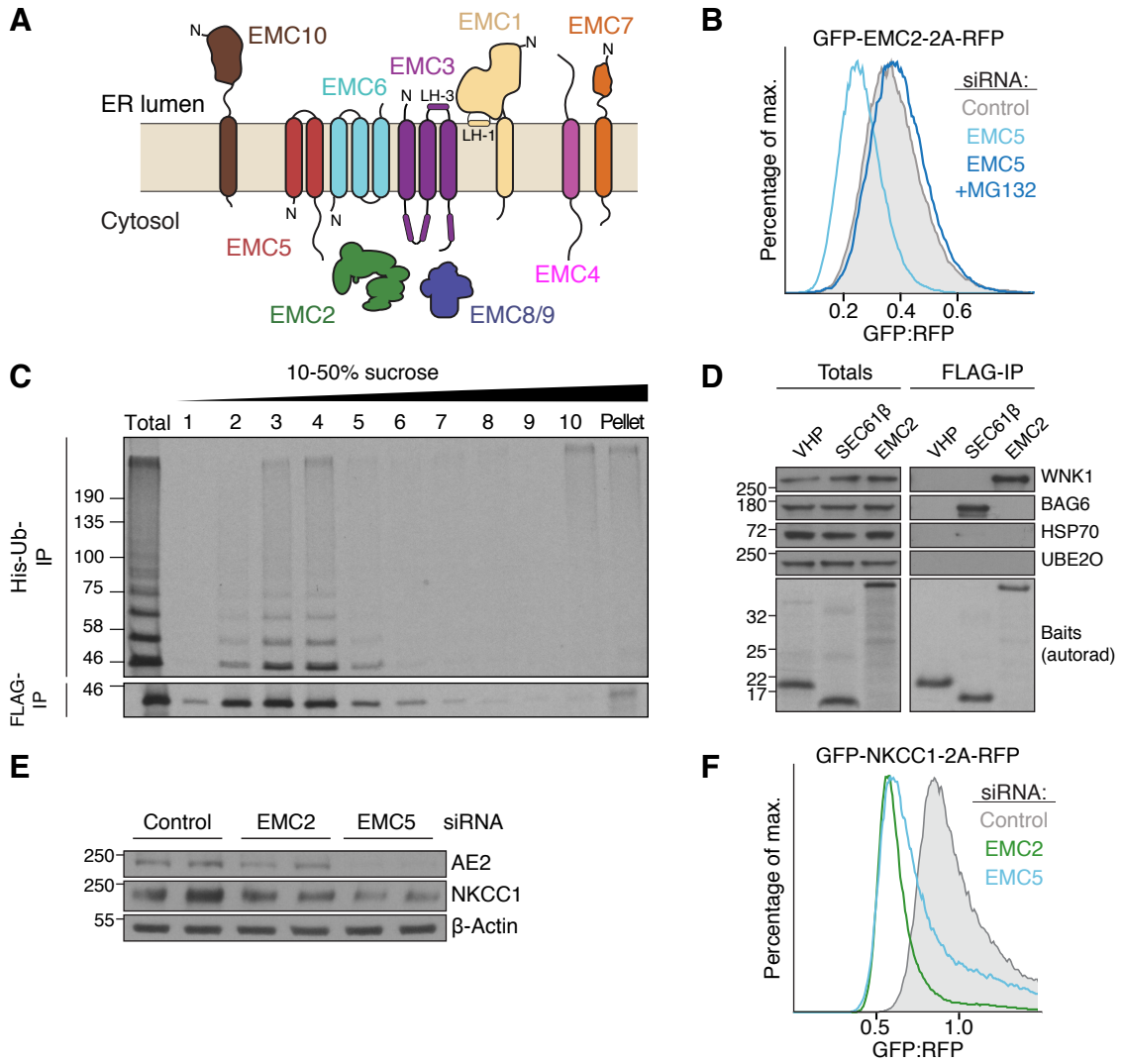

Figure S1. Unassembled EMC2 is recognized by the ubiquitin-proteasome pathway, Related to Figure 1.
(**A**) Topology of the EMC subunits based on the structure of the human EMC (Pleiner et al., 2020). Note that EMC8 and 9 are paralogues and their binding to EMC2, and thus incorporation into the EMC, is mutually exclusive. N = N-terminus; LH = Lumenal α-helix. (**B**) HEK293T cells stably expressing GFP-EMC2 were treated with either scrambled or EMC5 siRNA -/+ the proteasome inhibitor MG132. The relative levels of GFP-EMC2 normalized to an internal RFP expression control (see Method details) are plotted as a histogram and reflect changes in post-translational subunit stability. (**C**) Soluble unassembled EMC2 stably recruits E3 ubiquitin ligase activity. An *in vitro* translation reaction of ^{35}S-methionine-labeled 3xFLAG-EMC2 in rabbit reticulocyte lysate (RRL) was fractionated on a 10-50% (w/v) sucrose gradient. Unfractionated translation (Total) and all fractions were subjected to FLAG-IP and subsequently incubated with ATP, His-tagged ubiquitin (His-Ub), as well as E1 and E2 enzymes to detect the presence of co-purifying E3 ligase activity. Ubiquitinated species were enriched after elution from the FLAG resin by Ni^{2+}-chelate affinity chromatography. (**D**) Native FLAG immunoprecipitation of ^{35}S-methionine-labeled 3xFLAG-tagged chicken villin headpiece domain (VHP, an inert control), the tail-anchored protein SEC61β and EMC2 from RRL. Eluates were analyzed by SDS-PAGE and western blotting with the indicated antibodies. Baits were detected by autoradiography (autorad). WNK1 was originally discovered as the top hit of the EMC2 IP via mass spectrometry (Figure 1C, Table S2). (**E**) Endogenous NKCC1 expression relies on a functional EMC. HeLa cells were treated with either scrambled, EMC2, or EMC5 siRNA and harvested for western blotting with the indicated antibodies. Expression of endogenous AE2 (SLC4A2), a previously identified EMC substrate, displayed a similar EMC dependence. (**F**) NKCC1 is post-translationally destabilized in the absence of the EMC. HEK293T cells stably expressing GFP-NKCC1-2A-RFP were treated with either scrambled, EMC2, or EMC5 siRNA and the GFP:RFP ratio was analyzed by flow cytometry. The observed post-translational destabilization of NKCC1 upon EMC depletion is consistent with a requirement for EMC in NKCC1 biogenesis.

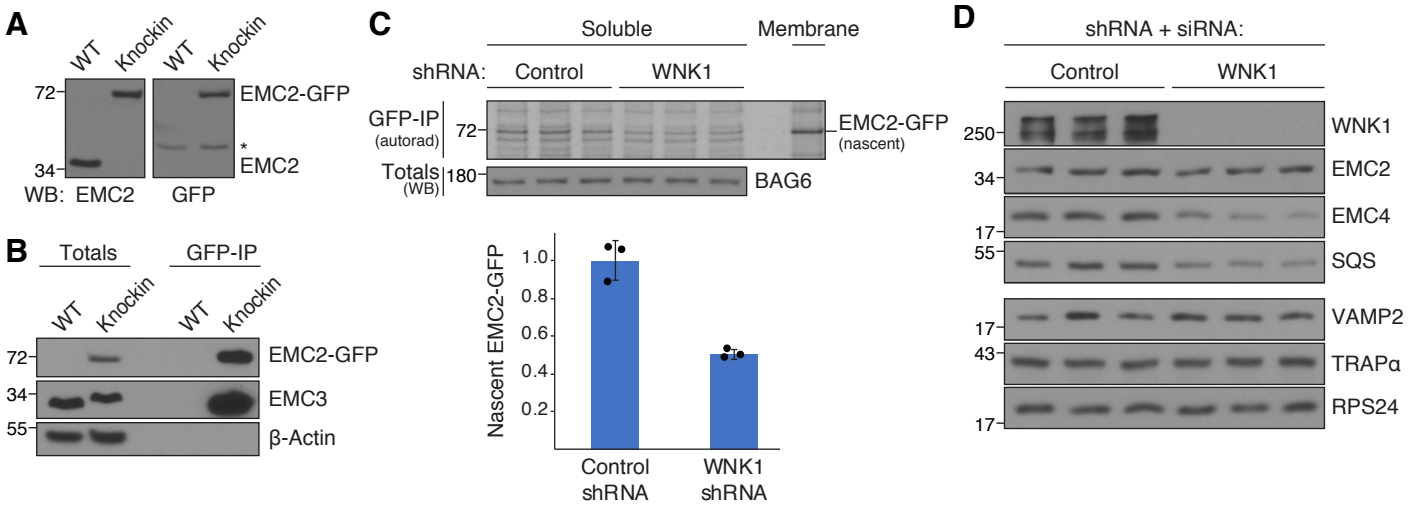

Figure S2. WNK1 knockdown destabilizes endogenous EMC subunits and substrates, Related to Figure 2.
(**A**) HeLa EMC2-GFP CRISPR knockin cells were lysed alongside HeLa wild type (WT) cells and analyzed by SDS-PAGE and western blotting (WB) with either EMC2 (left) or GFP (right) antibodies. The asterisk indicates a nonspecific band recognized by the GFP antibody. (**B**) HeLa EMC2-GFP CRISPR knockin and HeLa wild type (WT) cells were solubilized in lysis buffer containing 1% (w/v) LMNG and subjected to IP with an anti-GFP nanobody. Totals and eluates were analyzed by SDS-PAGE and western blotting with the indicated antibodies. (**C**) HeLa EMC2-GFP CRISPR knockin cells were transduced to stably express either scrambled or WNK1 shRNA in triplicate. Newly synthesized proteins were pulse-labeled with ^{35}S-methionine. Cells were then quenched with unlabeled methionine and mechanically lysed to yield a soluble cytosolic fraction (as in Figure 1D). Unassembled newly synthesized EMC2-GFP was then purified with an anti-GFP nanobody and analyzed by SDS-PAGE and autoradiography (autorad). A quantification of nascent unassembled EMC2-GFP is shown below. Error bars represent the mean and standard deviation of three independent replicates (n=3). (**D**) HeLa EMC2-GFP CRISPR knockin cells were transduced to stably express either scrambled or WNK1 shRNA in triplicate. After selection of shRNA-transduced cells with puromycin, cells were additionally treated with scrambled or WNK1 siRNA and then harvested for analysis of total levels of endogenous proteins by SDS-PAGE and western blotting with the indicated antibodies.

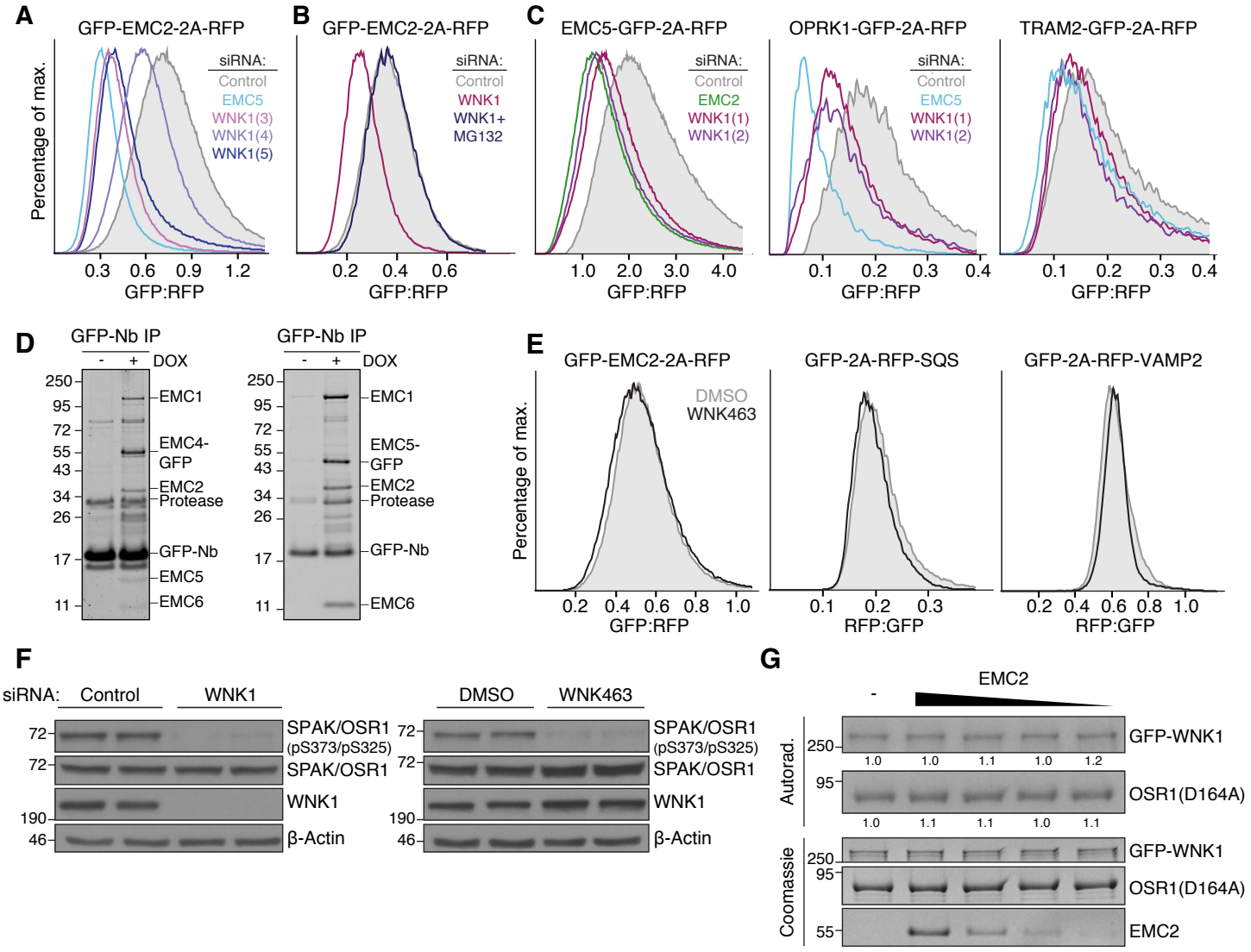

Figure S3. The kinase activity of WNK1 is not required for its role in EMC assembly, Related to Figure 2.
(**A**) HEK293T cells stably expressing GFP-EMC2-2A-RFP were treated with a scrambled control or EMC5 siRNA, or three independent WNK1 siRNAs for 72 hours. 24 hours following siRNA transfection the fluorescent reporter was induced for 48 hours. Cells were then analyzed by flow cytometry and their GFP:RFP ratio plotted as a histogram. (**B**) HEK293T cells stably expressing GFP-EMC2 were treated with either scrambled or WNK1 siRNA -/+ the proteasome inhibitor MG132 and analyzed as in (A). (**C**) Stable HEK293T cell lines expressing either (left) EMC5-GFP-2A-RFP or (middle) OPRK1-GFP-2A-RFP or (right) TRAM2-GFP-2A-RFP were treated with the indicated siRNAs for 72 hours. 24 hours following siRNA transfection the fluorescent reporter was induced for 48 hours. Cells were then analyzed as in (A). (**D**) HEK293T cell lines stably expressing (left) EMC4-GFP-2A-RFP, and (right) EMC5-GFP-2A-RFP, were harvested 48 hours after reporter induction, detergent-solubilized and subjected to IP with an anti-GFP nanobody (Nb). The eluates were analyzed by SDS-PAGE and Sypro Ruby staining. GFP-tagged EMC4 and 5 are incorporated into the intact EMC and thus co-purify endogenous EMC subunits, which were identified by mass spectrometry. The characterization of the HEK293T GFP-EMC2-2A-RFP cell line was described before (Pleiner et al., 2020). (**E**) Stable HEK293T cell lines expressing either (left) GFP-EMC2-2A-RFP, (middle) GFP-2A-RFP-SQS or (right) GFP-2A-RFP-VAMP2 were treated either with DMSO or 1 μM pan-WNK inhibitor WNK463 (IC_{50}= 5 nM for WNK1; Yamada et al., 2016) for 68 hours. 20 hours following drug treatment the fluorescent reporter was induced for 48 hours. Cells were then analyzed as in (A). No effect of WNK463 on EMC2 stability or EMC activity was observed. (**F**) Left: HEK293T cells were treated with either scrambled control or WNK1 siRNA for 72 hours. Right: HEK293T cells were treated with DMSO or 1 μM WNK463 for 68 hours. Cells were then harvested for Western blotting with the indicated antibodies. Note that WNK463 treatment reduced SPAK/OSR1 phosphorylation (pSer373 in SPAK, pSer325 in OSR1) much like a WNK1 knockdown. (**G**) EMC2 binding does not affect WNK1 kinase activity. Purified GFP-tagged WNK1 and its substrate kinase OSR1, with a kinase-dead mutation D164A, were incubated with ^{32}P-ATP in the presence or absence of increasing amounts of purified EMC2. Kinase reactions were analyzed by SDS-PAGE and autoradiography. No detectable change in WNK1 autophosphorylation or OSR1 phosphorylation could be observed. Further, no significant EMC2 phosphorylation was detected.

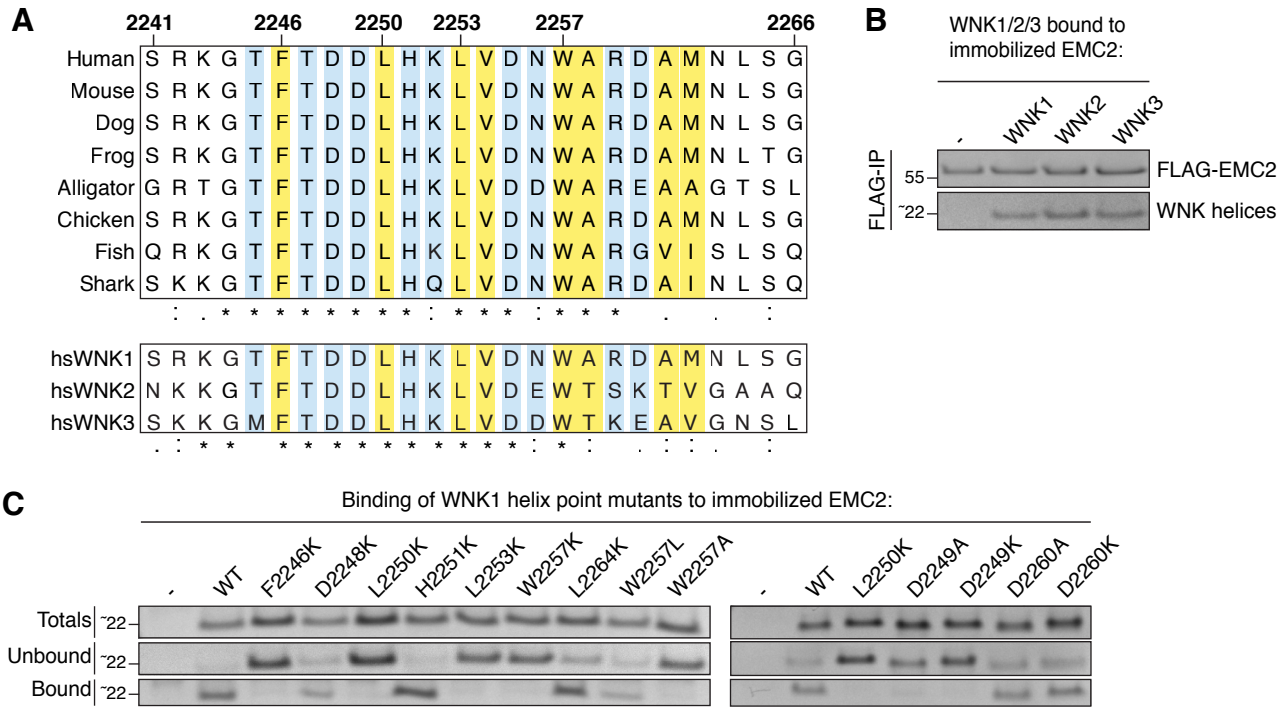

Figure S4.

WNK1 amphipathic α-helix is conserved and binds EMC2 via its hydrophobic face, Related to Figure 3.

(**A**) High sequence conservation of the WNK1 α-helix in vertebrates. Top: Shown is a sequence alignment corresponding to residues 2,241-2,266 in human WNK1. The following species were included: Human (*Homo sapiens*), Mouse (*Mus musculus*), Dog (*Canis lupus familiaris*), Frog (*Xenopus tropicalis*), Alligator (*Alligator sinensis*), Chicken (*Gallus gallus*), Fish (*Nothobranchius furzeri*) and Shark (*Callorhinchus milii*). Bottom: Alignment of human WNK1, WNK2 and WNK3 in the region corresponding to the amphipathic α-helix (residues 2,241-2,266 in human WNK1). WNK4 has no region with significant sequence homology. (**B**) The equivalent helical regions in WNK2 (residues 2,153-2,178) and WNK3 (residues 1,641-1,666) also interact with EMC2. Purified 3xFLAG-tagged EMC2 was incubated with either buffer or His_{14}-*bd*SUMO-tagged WNK helix variants and then subjected to FLAG-IP. Bound proteins were eluted with SDS-PAGE sample buffer and analyzed by SDS-PAGE and Coomassie staining. (**C**) The hydrophobic face of the WNK1 amphipathic α-helix interacts with EMC2. Purified 3xFLAG-tagged EMC2 was incubated with either buffer or His_{14}-*bd*SUMO-tagged WNK1 helix variants and then subjected to FLAG-IP. Bound proteins were eluted with SDS-PAGE sample buffer. Samples of totals, unbound, and elution were analyzed by SDS-PAGE and Coomassie staining.

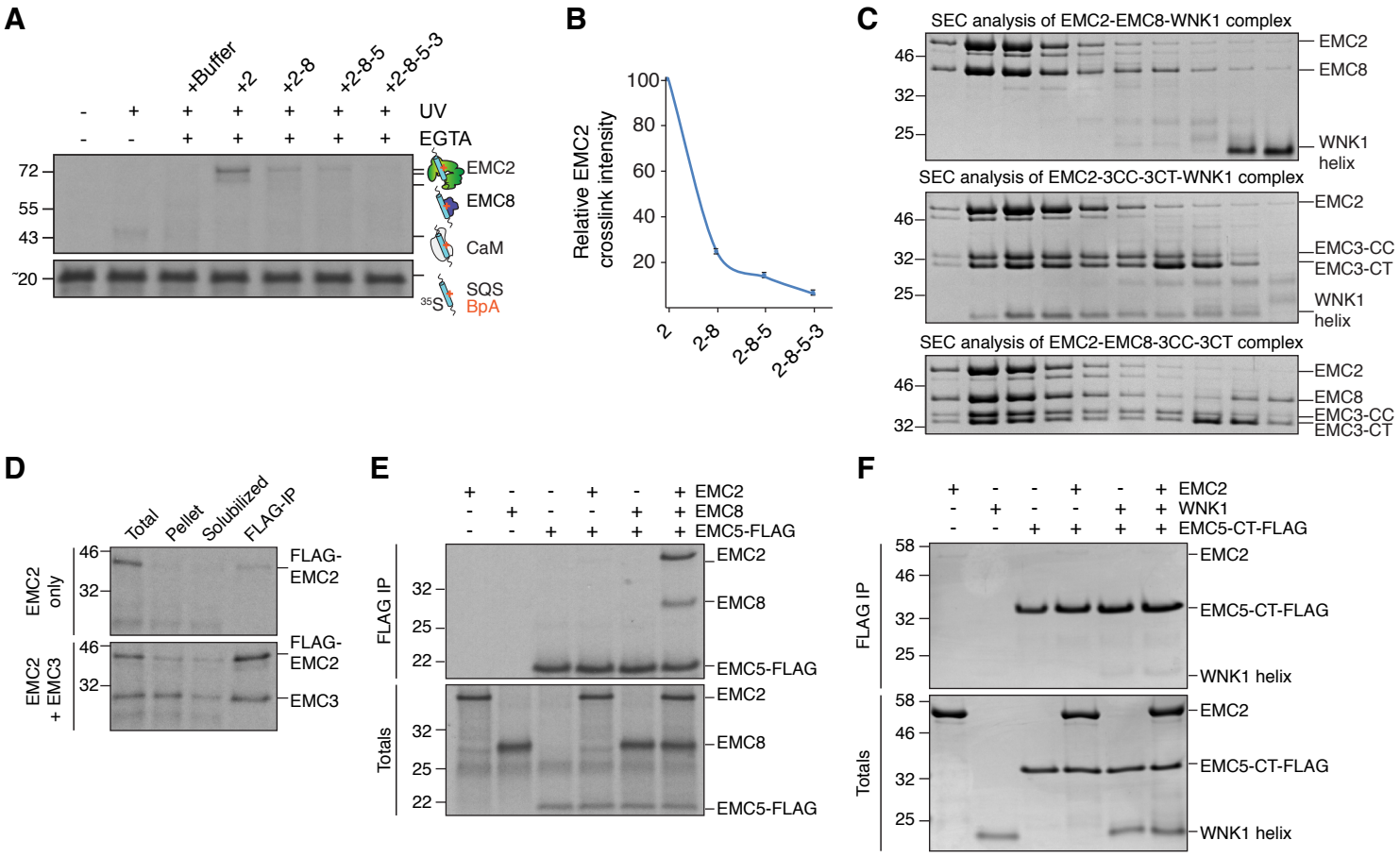

Figure S5. In vitro assembly of the cytosolic domain of the EMC, Related to Figures 4, 5 and 7.
(**A**) Same assay as in Figure 5A, but with the tail-anchored EMC substrate squalene synthase (SQS) containing a Bpa crosslinker in the position of amino acid Y400 in its transmembrane domain. (**B**) Quantification of the EMC2-SQS crosslink intensity from (A). Error bars represent the mean and standard deviation of three independent replicates (n=3). (**C**) Purified EMC2 was incubated with either (top) WNK1 helix and EMC8; (middle) WNK1 helix, EMC3 coiled coil (3-CC) and EMC3 C-terminus (3-CT) or (bottom) EMC8, 3-CC and 3-CT. The resulting complexes were analyzed by size-exclusion chromatography (SEC). Peak fractions were analyzed by SDS-PAGE and Coomassie staining. (**D**) EMC3 is sufficient to recruit EMC2 to the membrane. ^{35}S-methionine-labeled 3xFLAG-EMC2 was translated alone or with untagged EMC3 in RRL in the presence of canine rough microsomes (cRMs). cRMs were isolated by centrifugation through a sucrose cushion, resuspended, and solubilized in digitonin. The resulting detergent lysate was subjected to FLAG-IP and analyzed by SDS-PAGE and autoradiography. (**E**) EMC5 binds selectively to the EMC2-8 complex. Binding assays were performed as in (D) for full-length 3xFLAG-tagged EMC5 alone or in combination with 3xHA-tagged EMC2, EMC8, or both. (**F**) The EMC2-WNK1 complex is not capable of binding to the EMC5 C-terminus (5-CT). As in Figure 7B, using purified EMC2 and 5-CT, but with WNK1 helix instead of EMC8.

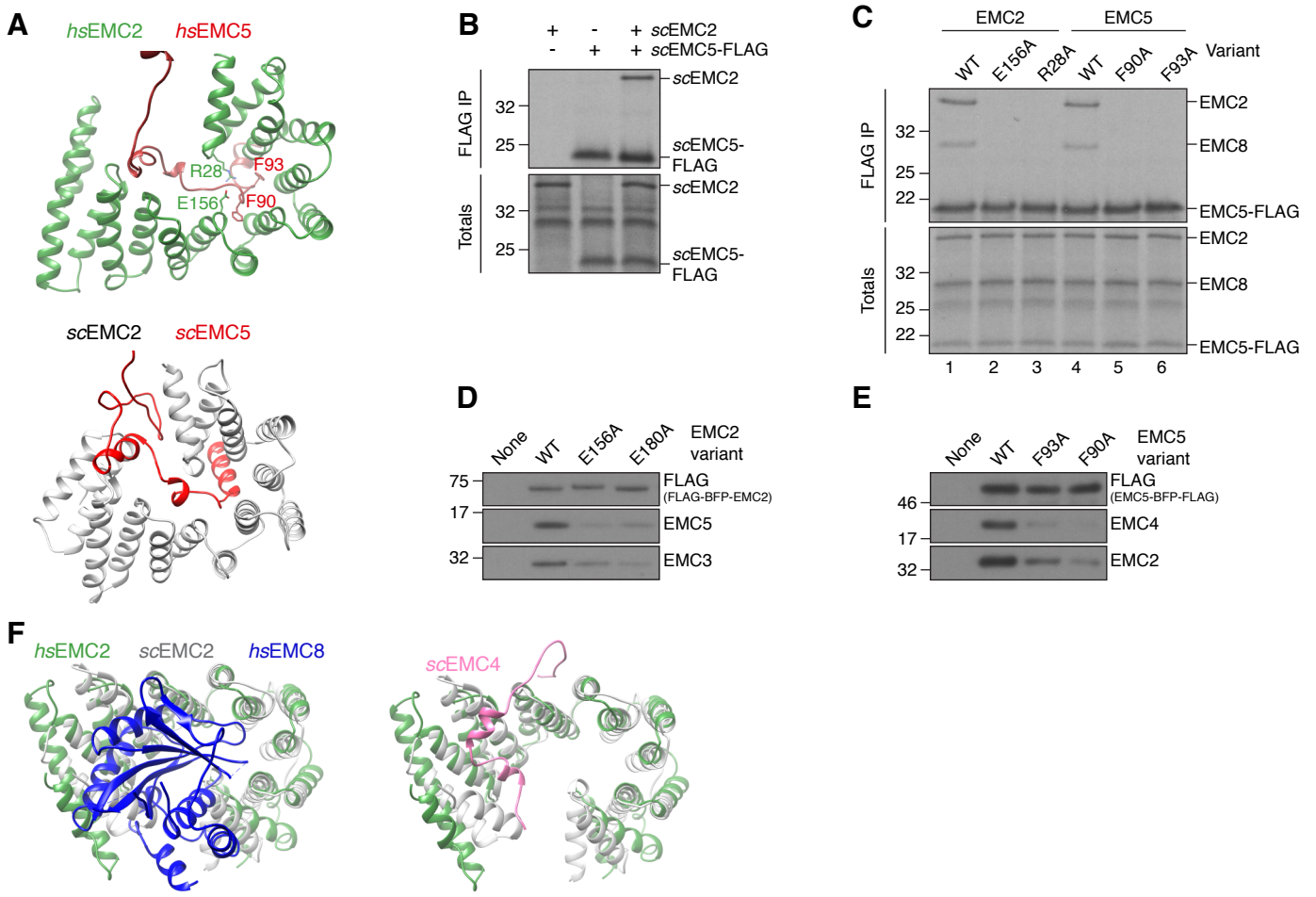

Figure S6. EMC2 is anchored at the membrane by interactions with both EMC3 and EMC5, Related to Figure 7. (**A**) Comparison of the yeast (PDB: 6WB9; Bai et al., 2020) and human EMC2-5 interfaces (PDB: 6WW7; Pleiner et al., 2020). In contrast to human EMC2, the N-terminus of yeast EMC2 packs against an elongated unstructured loop of yeast EMC5, resulting in a more extensive interface. Interface residues in human EMC2 and EMC5 that were mutated in (C-E) are highlighted. (**B**) In the absence of an EMC8 homolog in yeast, *S. cerevisiae* (*sc*) EMC2 binds *sc*EMC5 directly. Full-length *sc*EMC5-3xFLAG was translated alone or co-translated with 3xHA-tagged *sc*EMC2 in RRL in the presence of canine rough microsomes (cRMs). cRMs were isolated by centrifugation through a sucrose cushion, resuspended, and solubilized in digitonin. The resulting detergent lysate was subjected to FLAG-IP. Bound proteins were eluted with 3xFLAG peptide and analyzed alongside samples of total translation by SDS-PAGE and autoradiography. (**C**) Lanes 1-3: ^{35}S-methionine-labeled 3xFLAG-tagged wild type (WT) EMC5 was translated in the presence of 3xHA-tagged EMC2 (WT or the mutants E156A and R28A) and WT EMC8 in RRL in the presence of cRMs. Lanes 4-6: ^{35}S-methionine-labeled 3xFLAG-tagged EMC5 (WT or the mutants F90A and F93A) was translated in the presence of 3xHA-tagged WT EMC2 and WT EMC8 in RRL in the presence of cRMs. cRMs were pelleted from all translation reactions, solubilized in digitonin and the resulting lysate subjected to FLAG-IP. Analysis by SDS-PAGE and autoradiography. (**D**) HEK293T cells were transduced with lenti-viral vectors to stably express exogenous 3xFLAG-tagged WT or mutant EMC2 (E156A at EMC2-5, E180A at EMC2-3 interface, see Figure 4B). Cells were lysed and subjected to native FLAG-IP. Eluates were analyzed by Western blotting to determine the efficiency of assembly of WT and mutant EMC2, as judged by co-purification of EMC3 and EMC5. (**E**) As in (D), but with WT or mutant 3xFLAG-tagged EMC5. Both mutations are at the EMC2-5 interface. (**F**) In yeast, the analogous WNK1/EMC8 interface on EMC2 is occupied by the N-terminus of EMC4. A structural overlay of *S. cerevisiae* (*sc*) EMC2 (gray - PDB: 6WB9; Bai et al., 2020) and human EMC2 (green - PDB: 6WW7; Pleiner et al., 2020) is shown with human EMC8 (left, in blue) and *sc*EMC4 N-terminus (right, in pink). The N-terminus of EMC4 is poorly conserved, consistent with its non-essential role in binding EMC2 in metazoans.

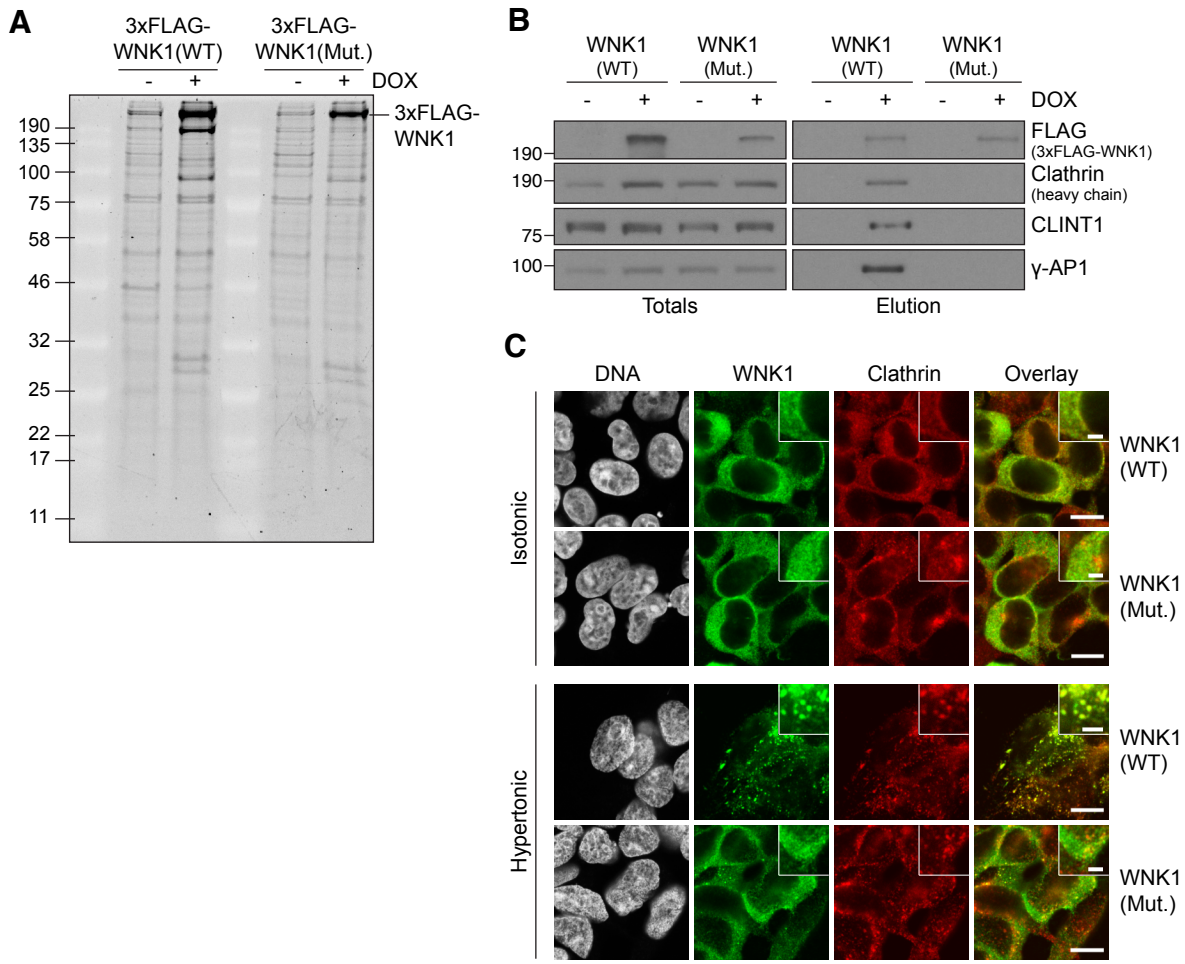

Figure S7. The WNK1 amphipathic helix mediates vesicle recruitment, Related to Figure 7.
(**A**) WNK1 co-immunoprecipitates with vesicle proteins. Stable HEK293T cell lines expressing 3xFLAG-tagged full length WNK1 wild type (WT) or WNK1 helix mutant (Mut = L2250K, L2253K, W2257K) were lysed mechanically under non-solubilizing conditions (as in Figure 1D, see Method details). WNK1 and its interacting proteins were immunoprecipitated from lysate with anti-FLAG resin. After elution with 3xFLAG peptide, the eluates were analyzed by SDS-PAGE and Sypro Ruby staining. Mass spectrometry revealed Clathrin and Clathrin-associated vesicle proteins as the highest specific hits of WT WNK1. Importantly, these interactions are sensitive to detergents commonly used for cell lysis (e.g. 1% [v/v] Triton-X-100 or NP-40), explaining why they were not observed before (Zagórska et al., 2007). Mutation of WNK1's amphipathic helix disrupts its interaction with vesicle-resident proteins. DOX = doxycyline. (**B**) Eluates from (A) were analyzed by western blotting with the indicated antibodies. (**C**) HEK293T cell lines stably expressing full-length 3xFLAG-tagged WT or Mut WNK1 were either left untreated (isotonic) or stimulated with 0.2 M Sorbitol for 5 min prior to fixation. Cells were then stained with DAPI (DNA), as well as anti-FLAG and Clathrin antibodies via indirect immunofluorescence and analyzed by confocal microscopy. Scale bars = 10 μm; Inset scale bars = 2.5 μm.

Table S1. EMC substrates involved in ion homeostasis/ cell volume regulation[a], Related to Figure 1.

Gene	Protein	Reference
SLC4A2	Anion Exchanger 2 (AE2)	(Shurtleff et al., 2018; Tian et al., 2019)
SLC6A6	Taurine transporter (TauT)	
ANO6	Anoctamine 6/ Small-conductance calcium-activated nonselective cation channel	
CLCN3	H(+)/Cl(-) exchange transporter 3	(Tian et al., 2019)
LRRC8E	Volume-regulated anion channel subunit LRRC8E	
SLC9A7	Sodium/hydrogen exchanger 7	
ATP2B1	Plasma membrane calcium-transporting ATPase 1	(Shurtleff et al., 2018)
ATP1A1/ ATP1B1	Sodium/potassium-transporting ATPase subunit alpha-1 / beta-1	(Satoh et al., 2015)

[a] This table lists previously identified EMC substrates (Satoh et al., 2015; Shurtleff et al., 2018; Tian et al., 2019) that are known regulators of cellular ion homeostasis and cell volume regulation (Jentsch, 2016).